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14. ABSTRACT
There is a critical need for markers of metastasis that will allow patients who are node negative to be separated into two groups: those who need further therapy and those who have been cured by their surgery. We hypothesized that changes in cellular biochemistry, that need not involve changes in the expression of particular genes, are important determinants of metastatic behavior, and we further hypothesized that many of these changes might be mimicked by the binding of an antibody with the right binging characteristics. We set out to test the idea that novel determinants of metastatic behavior could be identified by expressing single chain antibodies inside non-metastatic cells, and then screening those cells for the acquisition of a more metastatic phenotype. The antibody responsible for this increased metastatic ability would then be isolated from these cells and used to identify the protein with which it interacted. The project has met with significant technical challenges which have slowed progress. Nevertheless, significant progress has been made to overcome these difficulties and we anticipate being able to complete the study, having been granted a no-cost extension of one year.

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Table of Contents

Introduction:

Patients that die of breast cancer do so principally because of metatsiatic deposits of their disease. Thus, understanding the process of metastasis is an important goal in breast cancer research. There is a critical need for markers of metastasis that will allow patients who are node negative to be separated into two groups: those who need further therapy and those who have been cured by their surgery. Much has been learned about the role of proteases, growth, motility, angiogenic and survival factors, and other molecules involved in metastasis. Much, however, remains to be discovered, particularly about how the normal regulation of the activity of these proteins is disrupted during tumor progression. Attempts have been made in the past to identify new genes associated with metastatic behavior, but all of these attempts have shared the same weakness, in that they have only looked at changes at the level of gene expression. This ignores all the other way that the activity of a protein or biochemical pathway can be altered; changes in phosporylation state, changes in glycosylation, altered conformation, proteolytic cleavage, altered protein stability, altered sub-cellular localization, changes in enzymatic activity, etc.

In this study we hypothesized that changes in cellular biochemistry, that need not involve changes in the expression of particular genes, are important determinants of metastatic behavior, and we further hypothesized that many of these changes might be mimicked by the binding of an antibody with the right binging characteristics.

In this Exploration Award, we proposed to test the idea that novel determinants of metastatic behavior could be identified by expressing single chain antibodies inside non-metastatic cells, and then screening those cells for the acquisition of a more metastatic phenotype. The antibody responsible for this increased metastatic ability would then be isolated from these cells and used to identify the protein with which it interacted.

Body:

The goal of the Exploration Award mechanism was to allow investigators to explore untested concepts and develop novel testable hypotheses. The most important requirement for applications to this mechanism was that the proposals be novel and highly innovative. Implicit in this requirement and in the "high risk, high gain" philosophy of the mechanism is the recognition that the goals of the study may not be achievable as written and that the desired outcomes may not be forthcoming. In order to maximize the likelihood of success, we designed our studies such that, though highly novel and innovative, we could make use of established, rugged technology in which we had considerable experience. The novelty came from the way in which we combined these technologies and the untested concept underlying their application. We had in hand a synthetic antibody library that had been characterized and used successfully by others in published studies. The vectors we planned to use were already being used successfully in our lab, and the methods to bring these pieces together were standard techniques with which we had years of experience. We expected technical difficulties, but we expected them to be encountered in the screening aspects of the project – where the vast diversity

of the library that made it so useful would work against us since the screening approaches we planned to use were limited in terms of throughput. These challenges notwithstanding we believed that the 18 month time-line for the project would be sufficient to meet the goals of the study in terms of demonstrating the feasibility of the approach and the identification of some biochemically active antibodies. However, as we have described in our previous report, unexpected challenges bedeviled what we thought would be the easy part of the study and we requested a no-cost extension to provide additional time in order to complete the work. As described below, the technical challenges have continued and have again forced us to alter our approach. Although this is the final report for this award, the work will continue, and the support provided by this award has been critical to our development of what we still believe will be an extremely valuable approach. We will continue to recognize this support as the program proceeds.

Detailed report:

Initial strategy:

In order to generate a retroviral vector library required for this project we made use of an existing, highly diverse phage-based synthetic single chain antibody library. The plan was to transfer the DNA cassettes that codes for the single chain antibodies in the library from the phage display vector, in which the library was constructed, and to place them in a eukaryotic expression vector, thus recapitulating the library in a vector that would allow intracellular expression of the antibodies in cancer cells. This vector is a self contained, tetracycline regulable, retroviral construct which, we believed, would allow very efficient transfer of the library into the target cells. Transduced cells were then to be screened by a variety of *in vitro* and *in vivo* assays to identify clones with a more progressed phenotype, after which the causative antibodies were to be isolated and used to purify, identify and characterize the proteins with which they interacted. It was planned that within the first year of the study we would have constructed the vector library, transdced the cells, selected clones, isolated the antibodies and be conducting validation studies on the selected antibodies. The characterization of the antibodies was to be done in the remaining 6 months of the project. With hindsight, this was an ambitions schedule even without any technical difficulties.

Progress to date:

In order for the whole project to work it was critical that in shifting from one vector system to the other, none of the diversity present in the library be lost. We therefore planned two independent strategies by which the antibody cassette was to be transferred from the phage vector to the retroviral vector: 1) PCR based amplification of the cassette and 2) a restriction digestion based sub cloning method. In planning the study we suspected that the PCR based approach was going to be the easiest and most likely to maintain diversity. However, the library was originally constructed by a sub-cloning strategy using sub-cassettes flanked by restriction sites for rare, 8 base cutting enzymes. These sequences, therefore, contain fairly long runs of poly-C or –G which made designing PCR primers that would a) produce adequate amplification, b) maintain the

appropriate reading frame of the cassette to be transferred, c) minimize the inherent bias of a pool based PCR reaction and d) allow directional insertion into the retroviral vector very difficult. We spent a considerable amount of time refining the design of these primers, optimizing the amplification strategy and testing a variety of polymerases that allow us to use conditions that minimize the effects of the very GC rich primers that we had to use. During this process it became apparent that we needed methods that would allow us to evaluate the diversity of subsections of the library so that we could optimize our approach. This we did by a sampling-sequencing based approach and we discovered that amplification conditions had a very significant effect on the ultimate diversity of the pooled amplification products. This led us to re-design our strategy.

In parallel we had been developing a conventional restriction enzyme based subcloing approach, which progressed more smoothly. The down-side of this approach was that we could only use the enzymes used to generate the original library without risking the loss of diversity due to unintended digestion at sites within the cassette. This significantly limited our design flexibility and became more problematic when we made the decision to use an alternative vector as described below. This approach, however, has proved to be the most tractable and is the one used to construct our initial library, which seemed to adequately represent the diversity of the original library.

Our original plan had been to use a retroviral vector which we had successfully used before and which seemed to have the characteristics we required - pBSTR1. Unfortunately, as we started work on the project it rapidly became apparent that there were several issues with the construct that we had not appreciated and which would prove to be very problematic. This most significant was that we found it very difficult to reliably obtain high titer viral stocks using this construct. This was a big problem since we needed extremely high titer stocks in order for the screening assays to adequately sample the diversity of the library. As noted above the issue of the adequacy of the screens ability to sample the full diversity of the library was always the weak link of the project as we acknowledged in the initial proposal, and low titer viral stocks would have greatly exacerbated this problem. We, therefore, employed another self-contained retroviral vector – pLRT (PubMed ID 9175791). Shifting to this construct solved multiple problems – that of obtaining adequate titers, and further issues that we discovered with pBSTR1 - poor tetracycline regulability, and poor antibiotic slectability. The organization of the transcription cassettes in pBSTR1 is such that they interfere with each other in certain contexts. This causes problems with the efficacy of the selection marker and with the efficiency of tetracycline regulation. The vector we shifted to (pLRT), does not suffer from these problems since the cassettes are differently arranged and the vector makes use of an alternate selection marker – blasticidin rather than puromycin.

There is, however a down side to the use of this construct. The available restriction sites for the insertion of the gene to be expressed (in this case the synthetic antibody cassette) are extremely limited and not very convenient with respect to the constraints of the system we are using. This is not much of a problem for the more conventional use of the vector – to express one gene of interest, since a couple of simple steps allow almost any

insert to be cloned into the vector. This is not a practicable approach for the insertion of the antibody cassettes, firstly since we are very limited in the enzymes that we can use, and secondly since any additional step is extremely undesirable due to the deleterious effect of any additional step on the diversity of the ultimate library. As a result we had to modify the virus construct in order to allow us to use a simple one step subcloning process. This was done and the library was finally constructed. Assays of the diversity of the library by RFLP analysis suggests that it is still highly diverse.

Whilst we were working through the issues with the library construction we were developing the screening assays. As noted above, the issue of the ability of the screens to adequately evaluate the true diversity of the libraries has always been a tricky issue, since only a certain number of cells can be screened in each assay, and the total number of potential individual antibodies in the library is truly vast. Thus, it is very important to test the transduced cells as quickly after infection as possible since every cell doubling after infection effectively cuts in half the number of clones screened for a given number of cells assayed. We have, therefore, conducted the screens in two ways. In the first, the assays were conducted basically as described in the original application. The second approach was to first put the cells through a pre-screening assay a few hours after infection in order to remove productively infected cells where the antibody had not changed the biochemistry of the cell such that it acquired the phenotype we were screening for. Cells that were selected in these pre-screens, were then subjected to antibiotic selection and subsequently screened as we had originally planed.

Using this two tiered approach we were able in initial studies to identify cells that seemed to be more clonogenic, (soft agar assay), more invasive (Boyden chamber assay), more tumorigenic (nude mouse studies), and more metastatic (nude mouse studies), and that this behavior was modified by the absence/presence of doxycycline. In order to validate these potential hits, the top 10 candidates were expanded and the antibody cassettes cloned by PCR into an expression vector. The clones were then subjected to verification assays, to confirm that the phenotype for which they were screened was duplicated when re-transfected back into the parental cell line. The cassettes were also sequenced.

We were very disappointed to find that none of the ten potential hits were able to produce the phenotype for which they had been isolated when transfected into the parental cells. More disturbing was the fact that on sequencing we discovered that 3 or the 10 cassettes had stop codons in the reading frame of the antibody. Suspecting that this latter problem might be due to PCR induced mutagenesis, we re-cloned the cassettes from all 10 potential hits and verified their sequences. In all three cases we found the same in-frame stop codons. Clearly this is a major problem if these 10 clones are representative of the library as a whole. We were worried that the cloning strategy we had used to generate the library might have led to mutagenesis of the antibody cassettes in some way and so we went back to the original library and found that indeed many clones contained stop codons. We consulted with the colleague from whom we had obtained the library and he confirmed that they had run into a similar problem and it had become generally recognized that this was a problem with the original library that resulted from a flaw in the original construction of the library. He also informed us that he had obtained,

through a collaboration, a new library that they had personally validated (by screening of the library and isolating antibodies of interest), and which they were willing to provide to us. We have now independently validated the library in several ways, by sequencing numerous clones and by a biochemical screen that demonstrates that a very significant proportion of clones in the library can produce an antibody-like protein when introduced into cells. We are currently in the process of transferring this library into the retroviral construct and hope to be able to start screening shortly.

Given the issues with the original library, why were we able to isolate 10 clones that seemed to have acquired the phenotypes screened for, and why should these phenotypes be doxycycline regulable? We always anticipated that some of the clones that we would isolate would in fact be the result of insertional mutagenesis and the validation step was supposed to identify such clones for later analysis. That the phenotypes are doxycycline regulable is intriguing and suggests that the regulation of the promoter in the retrovirus must be altering the expression of some gene or altering the chromatin structure such that gene regulation is altered. We are in the process of deciding how to analyze these clones to understand their behavior and identify the genes responsible.

In conclusion, multiple technical challenges and unforeseen problems with key reagents have bedeviled this project. Nevertheless, we believe that the underlying concept is still sound and even though no longer supported by the DOD, we are continuing with the project and if and when we succeed we will gratefully acknowledge the support that made this project possible and allowed us to turn an idea into a valid area of research.

Key Research Accomplishments:

1) We have developed a strategy to develop highly diverse antibody libraries into mammary epithelial cells using a novel self-contained retroviral vector variant.

2) We have established and validated screening approaches that will allow us to screen for biologically active clones in our second generation antibody library.

3) We have isolated ten cell clones that have acquired doxycycline regulable phenotypic traits consistent with increased metastatic potential which should be valuable reagents with which to explore the determinants of metastatic behavior.

Reportable Outcomes:

None

Conclusions:

As noted above, this project turned out to have very significant technical challenges from unanticipated sources. Whilst this has been extraordinarily frustrating, the work has allowed us to learn a great deal about the issues involved in transferring highly diverse synthetic antibody libraries into novel vector systems. This information has been invaluable in our efforts to generate a second generation library. We have also learned a great deal about the systems that we will use to screen this new library, which we believe greatly enhances the likelihood that we will ultimately succeed. The support from the DOD has been critical to this endeavor and when we ultimately have results that we can report, that support will be recognized.